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Manganese Acquisition by *Lactobacillus plantarum*

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Lactobacillus plantarum has an unusually high Mn(II) requirement for growth and accumulated over 30 mM intracellular Mn(II). The acquisition of Mn(II) by *L. plantarum* occurred via a specific active transport system powered by the transmembrane proton gradient. The Mn(II) uptake system has a K_m of 0.2 μM and a V_{max} of 24 nmol mg^{-1} of protein min^{-1}. Above a medium Mn(II) concentration of 200 μM, the intracellular Mn(II) level was independent of the medium Mn(II) and unresponsive to oxygen stresses but was reduced by phosphate limitation. At a pH of 5.5, citrate, isocitrate, and *cis*-aconitate effectively promoted MN(II) uptake, although measurable levels of 1,5-[^{14}C]citrate were not accumulated. When cells were presented with equimolar Mn(II) and Cd(II), Cd(II) was preferentially taken up by the Mn(II) transport system. Both Mn(II) and Cd(II) uptake were greatly increased by Mn(II) starvation. Mn(II) uptake by Mn(II)-starved cells was subject to a negative feedback regulatory mechanism functioning less than 1 min after exposure of the cells to Mn(II) and independent of protein synthesis. When presented with a relatively large amount of exogenous Mn(II), Mn(II)-starved cells exhibited a measurable efflux of their internal Mn(II), but the rate was only a small fraction of the maximal Mn(II) uptake rate.

Most organisms need at least trace amounts of Mn(II), which is required for a number of important metabolic functions, including the Hill reaction (splitting of H_2O) of photosystem II, production of secondary metabolites such as the peptide antibiotics of *Bacillus* spp. (23, 28), sporulation (6, 7), and as a cofactor for several enzymes. These enzymes include glutamine synthetase (10), RNA polymerase (25), alkaline phosphatase (16), catalase (17), and superoxide dismutase (9). However, compared to iron or the macronutrients, relatively little is known about Mn(II) acquisition and function. Cells of *Escherichia coli* grow well on 10^{-8} M Mn(II) (24), in comparison to their iron requirements of several micromolar (20). Despite its very low Mn(II) requirements, *E. coli* was shown to possess a specific Mn(II) transport system with high temperature and metabolic energy dependence (24). This work was followed by the demonstration of high-affinity active Mn(II) transport in *Bacillus subtilis* (7), *Staphylococcus aureus* (21, 27, 29), and *Rhodopseudomonas capsulata* (14). In contrast, an Mn(II) acquisition system of relatively low affinity and specificity was reported earlier in *Saccharomyces cerevisiae* (15).

Many lactic acid bacteria contain very high intracellular Mn(II) levels (3). Recent findings indicate that the approximately 30 to 35 mM manganese present in cells of *Lactobacillus plantarum* acts as a scavenger of toxic oxygen species, particularly the superoxide radical anion (O_2^-), replacing the micromolar levels of superoxide dismutase found in virtually all other oxygen-tolerant organisms (2, 3, 9). This unique nonenzymatic function for high levels of Mn(II) suggested that *L. plantarum* would be a good organism in which to study the Mn(II) uptake process. *Lactobacillus* growth medium can easily be made growth limiting for Mn(II), permitting study of induction and limitation effects. The observed high rate of Mn(II) uptake suggested large numbers of Mn(II) receptors or transporters on *Lactobacillus* organisms. Although the cells are easily cultured and their nutritional requirements have been well studied (8, 12), there are no reports on Mn(II) acquisition by any members of the *Lactobacillaceae* or *Streptococcaceae*. The characteristics of the Mn(II) active transport system found in *L. plantarum* and reported here are consistent with efficient acquisition of Mn(II) from fermenting plant tissue or rumen habitats and shed more light on the unusual biochemistry of the lactic acid bacteria.

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MATERIALS AND METHODS

L. plantarum 14917 was obtained from the American Type Culture Collection, Rockville, Md. The lyophilized culture was grown, assessed for purity, and subcultured onto many small glucose-APT agar slants. After growth became visible, these slants were rapidly frozen and stored at −70°C. Each experiment employed a new frozen slant culture.

Cells were grown on APT complex medium (2, 8) containing, per liter: tryptone, 10 g; yeast extract, 7.5 g; glucose, Na_3-citrate, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; $MgSO_4$, 0.8 g; Na_2CO_3, 1.25 g; $MnSO_4$, 107 mg; and thiamine-HCl, 0.1 mg. Tween 80 was omitted, the pH was adjusted to 6.7, and glucose and $MnSO_4$ were added after autoclaving. Omission of $MnSO_4$ produced low-Mn(II) APT containing 1.0 to 1.8 μM Mn(II), derived from the tryptone and yeast extract. Broth cultures were grown in 40% filled Erlenmeyer flasks shaken at 116 rpm at 37°C. Culture growth was followed turbidimetrically in sidearm flasks. Cells were counted under the microscope with a hemacytometer and related to the turbidimetric measurements. The APT salts buffer (pH 6.7) had the same composition as APT medium but lacked glucose, tryptone, yeast extract, Na_3-citrate, thiamine-HCl, and $MnSO_4$.

Transport assays. Manganese uptake experiments were performed on Mn(II)-starved *L. plantarum* cells as follows. Cells from a 12 to 18-h APT agar plate culture were resuspended to an optical density at 600 nm of 0.10 to 0.15 in low Mn(II) APT broth. After shaking at 37°C for 12 to 14 h, this culture reached an optical density at 600 nm of 0.6 to 1.1, and 10 ml was inoculated into 100 ml of fresh low-Mn(II) APT. After 5 to 6 h at 37°C, growth in this flask tapered off at an optical density at 600 nm of 0.35 to 0.60 (1.1×10^8 to 2.3×10^8 cells per ml or 46 to 97 μg of cell protein per ml) due to Mn(II) limitation. These were washed and concentrated 20-fold by centrifugation (4,080 × *g* for 5 min) in the Mn(II)-

depleted medium or APT salts buffer and placed on ice. The supernatant medium was passed through a membrane filter (0.22-μm pore size), adjusted to pH 6.7, and used as Mn(II)-free assay medium. In some experiments, APT salts with glucose or a phosphate-free buffer with glucose replaced the spent medium. To measure Mn(II) uptake, 5 ml of the filtered Mn(II)-free spent medium or 5 ml of the appropriate buffered salts solution was placed in a 50-ml flask in a 37°C shaking water bath. Cells were added and allowed to equilibrate for 10 min, and then a mixture of $^{54}Mn(II)$ and $^{55}MnSO_4$ was added (20 to 50,000 dpm/ml). The number of cells added was adjusted to ensure that only a small fraction of the total Mn(II) was taken up during the assay. Duplicate 0.3-ml samples were removed and placed in wetted membrane filter tubes (0.45-μm pore size) in an Amicon VFM-3 filtration manifold under 380 mm Hg of vacuum. Each filter was immediately rinsed with 3 ml of low-Mn(II) APT medium or buffer and placed in a vial for counting. Controls included 0.3 ml of unfiltered cell suspension and filters through which cell-free $^{54}Mn(II)$-containing APT was passed. $^{54}Mn(II)$ was counted on a Beckman Gamma 8000 counter. Uptake experiments employing $^{109}Cd(II)$ were performed in an identical manner, but counted in a Beckman LS-8000 scintillation counter.

Total manganese content of cells and media was measured with a Perkin-Elmer model 703 atomic absorption spectrophotometer, employing both flame and graphite furnace assay modes. The protein content of cells was determined by the method of Lowry et al. (19) using bovine serum albumin as the standard (10^9 cells contained 0.42 mg of protein). Cell pellet volume in milliliters was determined by dividing the wet weight in grams of a known number of pelleted cells (10,000 × *g* for 5 min) by 1.08. Interstitial space in the pellet was estimated by dextran blue 2000 exclusion to be 40 to 50% of total pellet volume.

Electron microscopy was done with a Philips EM 300 by conventional techniques, as well as a Philips EM 400 with an energy dispersive X-ray microanalysis (EDAX) attachment. Cells in which manganese was to be localized were fixed in APT buffer containing 5% glutaraldehyde, embedded, and thin sectioned. The sections had to be floated off the ultramicrotome knife onto 10 mM phosphate buffer (pH 8.0) to prevent loss of the dark granules.

Media components were from Difco Laboratories, and salts and organic chemicals were from Fisher Scientific and Sigma Chemical Co. New England Nuclear Corp. (Canada) supplied $^{54}MnSO_4$ and $^{109}CdSO_4$.

RESULTS

Regulation and location of intracellular Mn(II). Once there was sufficient medium Mn(II) (200 μM) to allow the cells to accumulate approximately 32 mM, total intracellular manganese was independent of the medium manganese concentration and unaffected by growth under O_2, N_2, and air with 5 μM plumbagin (3, 11) in the medium (Fig. 1). In sharp contrast, cells grown on phosphate-limited APT accumulated only one-tenth their normal intracellular manganese (Fig. 1) (5). Interestingly, the reverse experiment, in which the normal level of medium phosphate (26.5 mM) was present but medium Mn(II) was very low (1.4 μM), also yielded cells deficient in both manganese and the numerous large polyphosphate granules always observed in APT-grown *L. plantarum* (Fig. 2C). Furthermore, unstained thin sections of cells showed that the electron-dense appearance of the granules is EDTA removable and could be restored by a few seconds of flotation of the section on an Mn(II)-containing

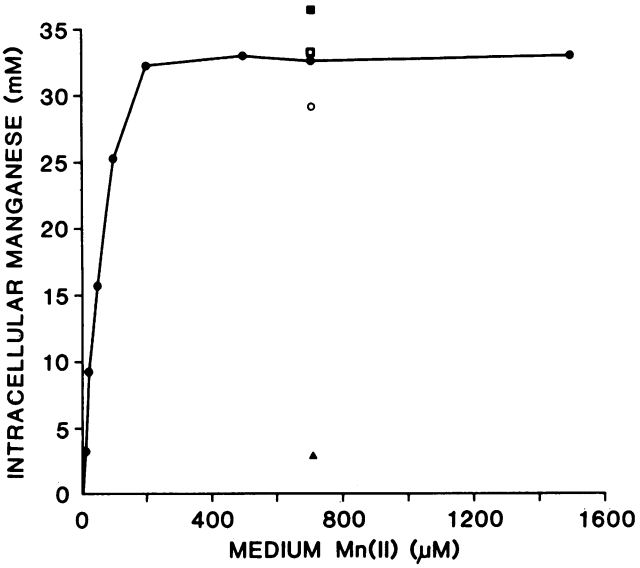

FIG. 1. Regulation of intracellular manganese in *L. plantarum*. Cells were grown in parallel flasks on APT medium varying only in Mn(II) content and harvested at a density of 1.75×10^9 to 2.15×10^9 cells per ml and a pH of 4.0 to 4.3, except for the lowest medium Mn(II) levels, which showed lowered growth extent and higher pH. Growth was in air (●), pure oxygen (○), air and 5 μM plumbagin (□), pure nitrogen (■), and air with low (0.5 mM) medium phosphate (▲).

solution (Fig. 2A and B). The presence of Mn(II) in thin sections showing electron-dense polyphosphate granules but not in EDTA-treated sections was demonstrated by using a Philips 400 electron microscope equipped with an energy dispersive X-ray microanalysis (EDAX) attachment (data not shown).

Kinetics of Mn(II) uptake. Active Mn(II)-starved cells were assessed for their initial Mn(II) uptake velocity (Fig. 3). Uptake rates were first order in respect to Mn(II) concentration from 1 to over 100 nM and nearly zero order over 1 μM. For the 100-fold Mn(II) increase between 1 and 100 μM, there was approximately a doubling of the $V_{initial}$, possibly due to a low-affinity transport system of the Mn(II)-Mg(II) type reported in yeasts (15). The calculated K_m of 0.2 μM for Mn(II) uptake by *L. plantarum* (Fig. 3, inset) is comparable to the K_m values obtained in other microorganisms (22). However, the V_{max} calculated for Mn uptake by *L. plantarum*, 10 μmol/10^{12} cells per min (23.8 nmol mg^{-1} of protein min^{-1}) is vastly greater than values reported for other bacteria (22). It should be noted that in different preparations of Mn(II)-starved cells, the observed V_{max} using 1.0 μM Mn(II) varied from 12.3 to 31 nmol Mn(II) mg^{-1} of protein min^{-1}.

Specificity of Mn(II) uptake. The cation selectivity of the Mn(II) uptake system of *L. plantarum* was investigated by exposing Mn(II)-starved cells to 0.1 μM $^{54}Mn(II)$ combined with 10 μM of each of a series of metal cations. The same assay format as for Fig. 3 was used, with Mn uptake assessed at 30 s and 1, 2, 5, and 10 min after exposure of the cells to the competing ions. The following cations were employed: Cr(III), Cu(II), Fe(II), (III), La(III), Co(II), Mg(II), Zn(II), Ca(II), and Cd(II). Of these cations, in 100-fold excess over Mn(II), only Cd(II) had a significant effect on the rate or extent of ^{54}Mn uptake, and Cd(II) inhibited both by >99%. The toxicity of each cation for *L. plantarum* was determined by placing 10 μl of a 1 mM solution of the

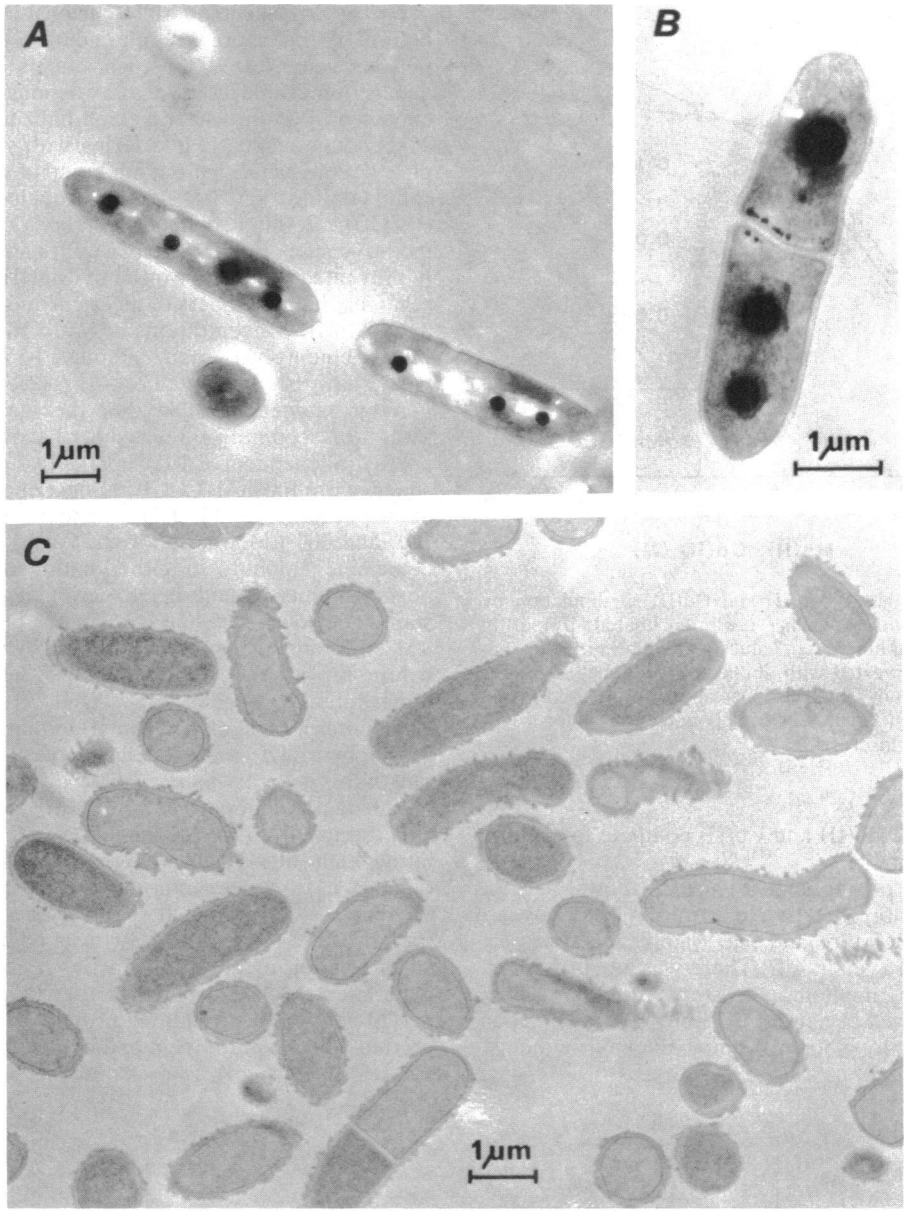

FIG. 2. Electron micrographs of thin sections of late-log-phase *L. plantarum*. The cells in (A) and (B) were grown in APT broth [710 μM Mn(II)], fixed in 5% glutaraldehyde, sectioned, and left unstained. Growth in low-Mn(II) (1.4 μM) APT broth yielded cells showing no large granules, even with lead citrate staining (C).

ion into a 4-mm well in an APT agar plate [710 μM Mn(II)] seeded with a lawn of cells. Only Cd(II) showed inhibitory activity, producing a 1.4-cm^2 zone of no growth around the Cd(II)-containing well.

By using the radioisotope ^{109}Cd(II), the ability of *L. plantarum* to take up Cd(II) was examined and compared to Mn(II) acquisition (Fig. 3). Unlike Mn(II), there appeared to be no saturating concentration of Cd(II) for maximal uptake velocity, and consequently no K_m could be calculated.

In another experiment, the effect of Mn(II) starvation of the cells on their Cd(II) and Mn(II) uptake rates was assessed. Cells grown on either 710 or 1.4 μM Mn(II)-containing APT were washed, resuspended in Mn(II)-free APT medium, and incubated with 1.0 μM ^{54}Mn(II) or 1.0 μM ^{109}Cd(II) for 5 min. Less than 1% of either cation became associated with the Mn(II)-sufficient cells, but 95% of the Cd(II) and 96% of the Mn(II) were taken up by the same number of cells grown in 1.4 μM Mn(II). Thus, Mn(II) starvation induced rapid Mn(II) and Cd(II) uptake.

To determine whether the observed inhibition of Mn(II) uptake by Cd(II) was truly competitive or due to the noncompetitive toxic effects of Cd(II), plots of the Cd(II) inhibition of Mn(II) uptake (Fig. 4A) and the Mn(II) inhibition of Cd(II) uptake (Fig. 4B) were performed. Preliminary results showed that when Mn(II) and Cd(II) were added to an uptake assay on an equimolar basis (0.1 μM), >95% of the initial uptake was of Cd(II) (data not shown). This was confirmed by the K_i values derived from Fig. 4, approximately 6 μM for Mn(II) inhibition of Cd(II) uptake and 0.9 μM for Cd(II) inhibition of Mn(II) uptake. The data in Fig. 4

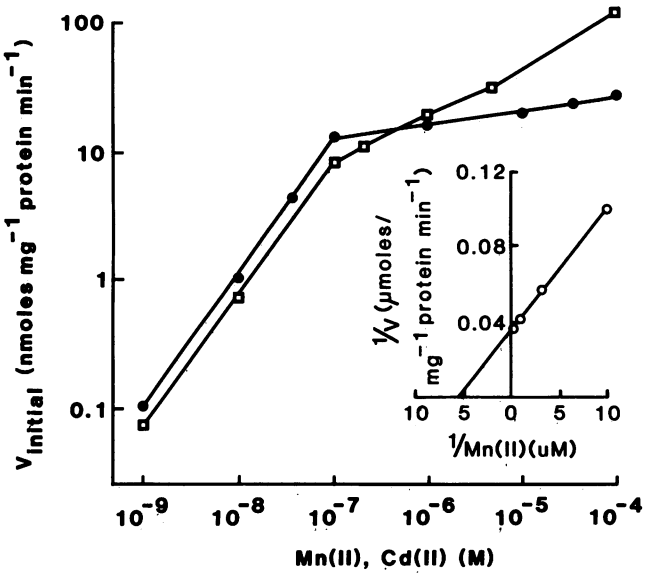

FIG. 3. Kinetics of initial Mn(II) and Cd(II) accumulation by Mn(II)-starved *L. plantarum*. Cells were preincubated (10 min) in spent, filtered APT medium. $V_{initial}$ values were determined on 0.3-ml portions of cells harvested with duplicate filter tubes 30 s after radiolabeled Mn(II) (●) or Cd(II) (□) was added to the 5-ml assay. The apparent saturation kinetics of Mn(II) uptake are shown in a double reciprocal plot (inset).

strongly suggest that Mn(II) and Cd(II) compete for uptake by a common system.

Temperature and pH optima. Figure 5 shows that *L. plantarum* is remarkably effective in acquiring Mn(II) over a broad range of temperature and pH. Significant uptake of Mn(II) was observed even at 4°C. There was a pH optimum from pH 5 to 7 in APT medium, consistent with an organism capable of growing well at neutral pH but releasing copious lactic acid and normally isolated from acidic environments.

Energy dependence of Mn(II) acquisition. To determine the nature of the energy requirement, a series of metabolic poisons were tested for their effects on 0.1 μM Mn(II) acquisition (Table 1). Cyanide was ineffective, as would be expected in an organism lacking both heme and iron (1, 2, 26). Sodium arsenate, interfering with substrate-level phosphorylation, caused partial inhibition of uptake. Tetrachlorosalicylanilide (TCS), *N, N'*-dicyclohexyl-carbodiimide (DCCD), 2,4-dinitrophenol, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone (CCCP), and nigericin completely prevented uptake of Mn(II) by Mn(II)-starved cells. With relatively large numbers of nigericin and TCS-poisoned cells and ^{54}Mn(II) concentrations down to 10^{-9} M, no detectable Mn(II) binding was seen, although 10 Mn(II) ions per cell would have been detected.

As it seemed possible that the lack of Cd(II) uptake saturation seen in Fig. 3 was due to a low-affinity, metabolic energy-independent Cd(II) uptake system, cells were poisoned with 100 μM CCCP as described for Table 1, and uptake of Mn(II) and Cd(II) in concentrations of 0.1, 2.0, 10, 100, and 300 μM was followed. At no concentration were detectable amounts of either cation associated with the poisoned cells. In another experiment unpoisoned Mn(II)-starved cells were treated with 10 μM bovine trypsin and 10 μM streptomycete protease (Sigma type XIV) for 10 min before the assay. Neither affected the rate or extent of Mn(II) uptake.

Short-term regulation of Mn(II) uptake. The rate of Mn(II) uptake by Mn(II)-starved cells decreased with time, and the rate of this decrease increased rapidly with the accumulation of substantial amounts of Mn(II) in the cells (Fig. 6). The effect seemed to be dependent on the concentration of intracellular Mn(II) as active uptake of low levels of Mn(II) (e.g., 10 nM) proceeded at a relatively constant velocity for the entire assay period. It should be noted that the concentration of cells in these assays was adjusted so that only a small fraction of the total Mn(II) was taken up during the experiment. To determine whether this decreasing rate of net Mn(II) uptake required protein synthesis, cells were pretreated with puromycin and exposed to 10 μM ^{54}Mn(II).

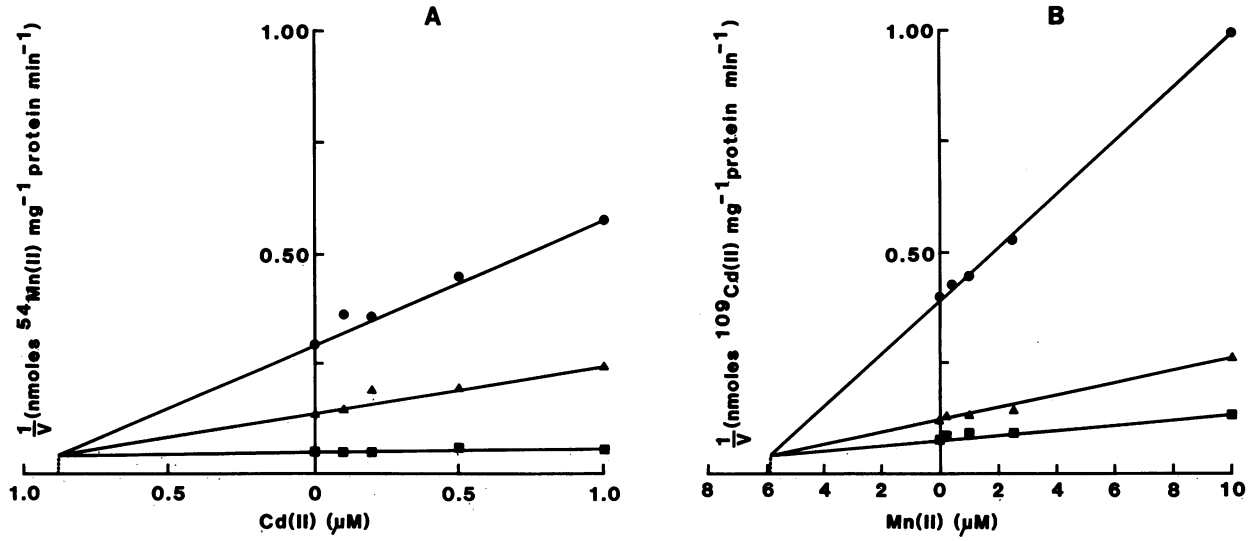

FIG. 4. Determination of the K_i of Cd(II) for ^{54}Mn(II) uptake (A) and the K_i of Mn(II) for ^{109}Cd uptake (B) in Mn(II)-starved cells. Assays were performed as described in the legend to Fig. 3, but with uptake assessed 1 min after simultaneous addition of the radiolabeled and unlabeled cations to the preincubated cells. (A) ●, 0.1 μM ^{54}Mn(II); ▲, 0.2 μM ^{54}Mn(II); ■, 2.0 μM ^{54}Mn(II). (B) ●, 0.2 μM ^{109}Cd(II); ▲, 1.0 μM ^{109}Cd(II); ■, 2.5 μM ^{109}Cd(II).

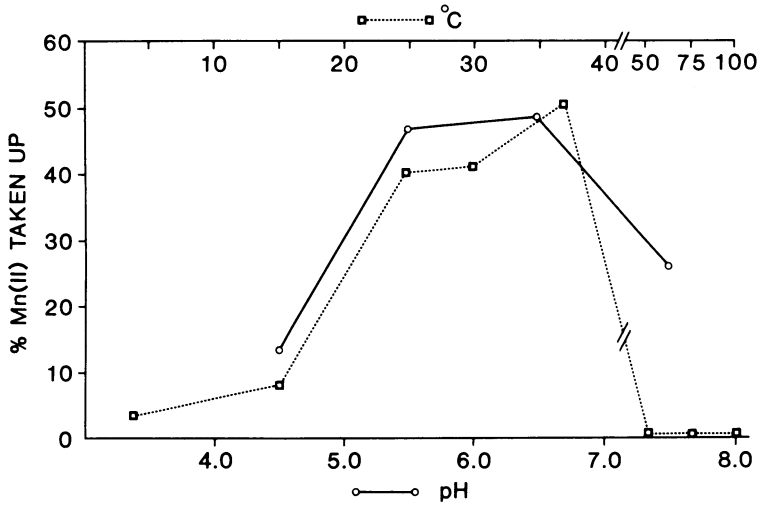

FIG. 5. Temperature (□) and pH (○) dependence of Mn(II) acquisition. Mn(II)-limited cells were exposed to 0.1 μM ^{54}Mn(II), and the percent Mn(II) taken up after 10 min was assessed. Preincubation of the cells (10 min) before the addition of Mn(II) was at the assay pH and temperature. All pH trials were at 37°C, and all temperature trials were at pH 6.7.

The antibiotic had no effect on the time-dependent Mn(II) uptake rate decrease (data not shown).

Forms of Mn(II) available to *L. plantarum*. The APT medium used for growth and Mn(II) uptake experiments contained 17 mM citrate. The ability of millimolar citrate at or near neutral pH to complex Mn(II) (4, 5) suggested that most medium Mn(II) was in a citrate complex and hence may have been taken up by *L. plantarum* from or as part of such a complex. Another possibility was that the cells might produce and release a soluble Mn(II) chelator analogous to the iron-binding siderophores released by many organisms (20) or the peptide Mn(II) chelator reported for *B. subtilis* (22). To test the latter hypothesis, the ability of *L. plantarum* to acquire Mn(II) from the spent, filtered APT used for the uptake experiments was compared to the ability of the cells to acquire Mn(II) from fresh low-Mn(II) APT medium. After correction for the available Mn(II) present in the low-Mn(II) fresh medium, there was no significant difference in the initial rate or extent (after 10 min) of Mn(II) uptake between the fresh and spent medium (data not shown). If there was no specific substance produced by the cells, could citrate or some of the other organic acids commonly associated with fermenting plant tissue and *L. plantarum* growth facilitate Mn(II) uptake? The Mn(II) uptake assay system was simplified by replacing the spent APT with APT salts buffer containing 55 mM glucose, and the effect of various additions and modifications to this medium on Mn(II) uptake was assessed (Table 2). Spent APT gave substantially faster Mn(II) uptake than the salts alone, but this difference was reduced by adding the medium level of citrate to the salts (Table 2). The removal of citrate, phosphate, and carbonate and the addition of 10 mM PIPES [piperazine-*N*, *N'*-bis (2-ethanesulfonic acid)] and KCl yielded a phosphate-free buffer in which presumably nearly all the Mn(II) was in the hexaquo free ion state. This Mn(II) appeared to be less available to the cells (Table 2).

TABLE 1. Effect of various substances on the uptake of Mn(II) by Mn-starved cells

Treatment[a]	Final concn	Mn uptake (% inhibition)[b]
No addition		0
Ethanol	36 mM	<1
KCN	1 mM	<1
Na_2HAsO_4	10 mM	21.3 ± 4.4
Valinomycin	10 μM	<1
Valinomycin	100 μM	20.9 ± 2.1
Nigericin	10 μM	>99
DCCD	100 μM	>99
CCCP	100 μM	>99
TCS	10 μM	>99
2,4-Dinitrophenol	2 mM	>99
Monensin	100 μM	>99
Gramicidin	100 μM	39.5 ± 1.3

[a] Ethanol, used as a solvent for all substances except KCN and Na_2HAsO_4, was added to the cells (10 μl per 5-ml assay).

[b] Mn(II)-starved cells were preincubated for 10 min in the presence of the added substance, and uptake was assessed by using the standard experimental format after 5 min of exposure to 0.1 μM ^{54}Mn(II). In the absence of any treatment, the cells took up 27 nmol of Mn(II) mg^{-1} (cell protein) min^{-1}.

Many organic acids rendered the 0.1 μM Mn(II) more available to the starved cells (Table 2). In most of those assay mixtures containing 20 mM organic acid, the presence or absence of phosphate had little effect on Mn(II) uptake. Although a wide variety of buffered organic acid solutions provided good Mn(II) availability at pH 6.7, at pH 5.5 *L. plantarum* cells could efficiently obtain Mn(II) only from buffer containing citrate or related tricarboxylic acids (Table 2). Employing [1-^{14}C]citrate and unlabeled Mn(II), essentially none of the citrate was taken up with the Mn(II) (data not shown).

Exit of Mn(II) from cells. Active cells containing high, moderate, or low levels of Mn(II) and resuspended in APT buffer and glucose or APT medium showed no detectable net loss of Mn(II) even over periods of several hours. However, the addition of 10 μM nonradioactive Mn(II) to the cells resulted in ^{54}Mn loss (Fig. 7). In the 1 μM Mn(II)-grown cells, the V_{max} of exit averaged 0.20 nmol mg^{-1} of protein min^{-1} over the first minute after a 10 μM addition. Since the data in Fig. 3 were obtained under the same conditions as those in Fig. 7A but with the medium Mn(II) radiolabeled

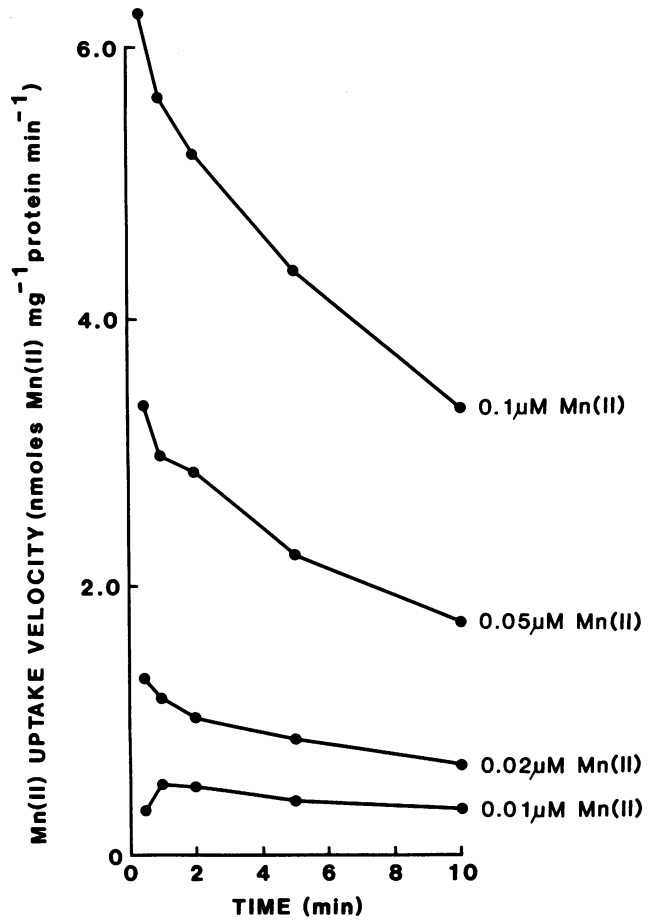

FIG. 6. Time-dependent changes in Mn(II) uptake rates of Mn(II)-starved cells. Cells were exposed to various concentrations of ^{54}Mn(II), and the rate of Mn(II) uptake was followed through time. Each rate was the calculated average for the period from the previous sampling to the present one. Time represents minutes after the cells were exposed to ^{54}Mn(II).

instead of the intracellular manganese, the two figures can be compared, i.e., the average $V_{initial}$ of uptake (1 min) by Mn(II)-starved cells from an added 10 μM Mn(II) was 16.2 nmol mg^{-1} of protein min^{-1}, and the $V_{initial}$ of exit (at 1 min) was 0.20 nmol mg^{-1} of protein min^{-1}. Therefore, the total initial uptake velocity was not greatly affected by the transient exit of Mn(II) ions. Cells grown in 6 μM Mn(II)-containing APT contained 4.4 mM manganese and showed a $V_{initial}$ of exit for the first minute after the 10 μM Mn(II) addition of 0.52 nmol mg^{-1} of protein min^{-1} (Fig. 7). The $V_{initial}$ of exit of 56 μM ^{54}Mn(II)-grown cells could not be calculated, as there was no detectable ^{54}Mn(II) loss by these cells when 10 μM ^{55}Mn(II) was added.

Formaldehyde (1%) and glutaraldehyde (5% [vol/vol]) were both shown to "seal" the cells, completely preventing the uptake or exit of Mn(II) (data not shown). In fact, washed cells with 32 mM internal Mn(II) suspended in APT salts buffer containing 5% glutaraldehyde showed retention of >98% of this Mn(II) over a 3-month period at 4°C. Toluene (1% [vol/vol]) added to cells has been shown to disrupt membranes (13, 24). When it was added to a suspension of active *L. plantarum* cells, over 90% of their internal Mn(II) was rapidly released (Fig. 7B). Although 1% formaldehyde prevented exit of Mn(II) from Mn(II)-treated cells, pretreament of toluenized cells with formaldehyde did not lessen the Mn(II) exit rate or extent (data not shown).

DISCUSSION

The unusually high manganese content of *L. plantarum* cells was generated by a specific high-affinity, high-velocity Mn(II) uptake system. The calculated K_m of 0.2 μM for Mn(II) uptake by *L. plantarum* (Fig. 3, insert) is comparable to values calculated for other bacteria: *E. coli*, 0.2 μM; *B. subtilis*, 1.0 μM; *R. capsulata*, 0.5 μM; and *S. aureus*, 2.0 μM (22). However, as might be expected from its very high Mn(II) requirements and content, the rate of uptake by *L. plantarum* was greater than that of the other bacteria studied. *E. coli*, *B. subtilis*, *S. aureus*, and *R. capsulata* are reported to have initial V_{max} values of 1 to 12 nmol of Mn(II) per 10^{12} cells per min^{-1} (22). *B. subtilis* showed a 50-fold increase in its V_{max} for Mn(II) uptake when derepressed for sporulation, but this still resulted in a V_{max} nearly 100 times slower than that observed in *L. plantarum*. Due to the absence of any binding of Mn(II) to the Mn(II) receptors of poisoned *L. plantarum* cells, the number of binding sites was not determined; thus, it was not determined whether the high V_{max} of *L. plantarum* was due to more Mn(II) transporters or to Mn(II) transporters with a faster turnover time than those in other microorganisms.

There is considerable evidence indicating that *L. plantarum* and related lactic acid bacteria contain millimolar

TABLE 2. Mn(II) uptake from various media

Uptake assay medium[a]	% Mn(II) taken up[b]	
	pH 5.5	pH 6.7
Spent APT broth[c]	52 ± 5.1	60 ± 7.9
+ Na_3-citrate		34 ± 3.4
APT salts + 55 mM glucose	4.3 ± 0.8	21 ± 4.7
+ Na_2CO_3		21 ± 2.1
Phosphate-free buffer[d]		2.6 ± 1.4
+ 0.4 mM K_2HPO_4		2.8 ± 0.8
+ 2.0 mM K_2HPO_4		2.4 ± 0.8
+ 10 mM K_2HPO_4		4.8 ± 2.0
+ 20 mM K_2HPO_4		33 ± 6.6
+ 20 mM citrate	20.7 ± 1.1	19.4 ± 2.3
+ 20 mM isocitrate	21.1 ± 2.0	16.8 ± 3.1
+ 20 mM *cis*-aconitate	9.4 ± 1.0	21.6 ± 2.3
+ 20 mM nitrilotriacetate	<0.1	0.8 ± 0.2
+ 20 mM EDTA	<0.1	<0.1
+ 20 mM α-ketoglutarate	<0.1	22.8 ± 1.9
+ 20 mM succinate	<0.1	22.0 ± 2.4
+ 20 mM fumarate	<0.1	18.1 ± 3.0
+ 20 mM malate	<0.1	20.3 ± 2.7
+ 20 mM oxaloacetate	<0.1	24.6 ± 2.2
+ 20 mM tartarate	<0.1	17.7 ± 3.1
+ 20 mM oxalate	<0.1	18.5 ± 1.9
+ 20 mM acetate	<0.1	23.3 ± 4.0
+ 20 mM lactate	<0.1	2.6 ± 3.1
+ 20 mM pyruvate	<0.1	1.3 ± 2.6
+ 20 mM butyrate	<0.1	17.2 ± 2.1
+ 20 mM iminodiactetate	<0.1	9.9 ± 1.9
+ 20 mM pyrophosphate	2.6 ± 1.1	15.1 ± 1.4

[a] Mn(II)-starved cells were cultured and assays were performed as described in the legend to Fig. 3.

[b] The percent of total added 54 Mn(II) (0.1 μM) taken up by the cells after 10 min of incubation. Values are the average of duplicate or triplicate assays normalized to the same number of cells.

[c] The filtered supernatant fluid of the Mn(II)-limited growth medium of the cells employed.

[d] Phosphate-free buffer contained 10 mM PIPES, 56 mM KCl, 86 mM NaCl, 13.5 mM $MgCl_2$, and 55 mM glucose (pH 5.5 or 6.7).

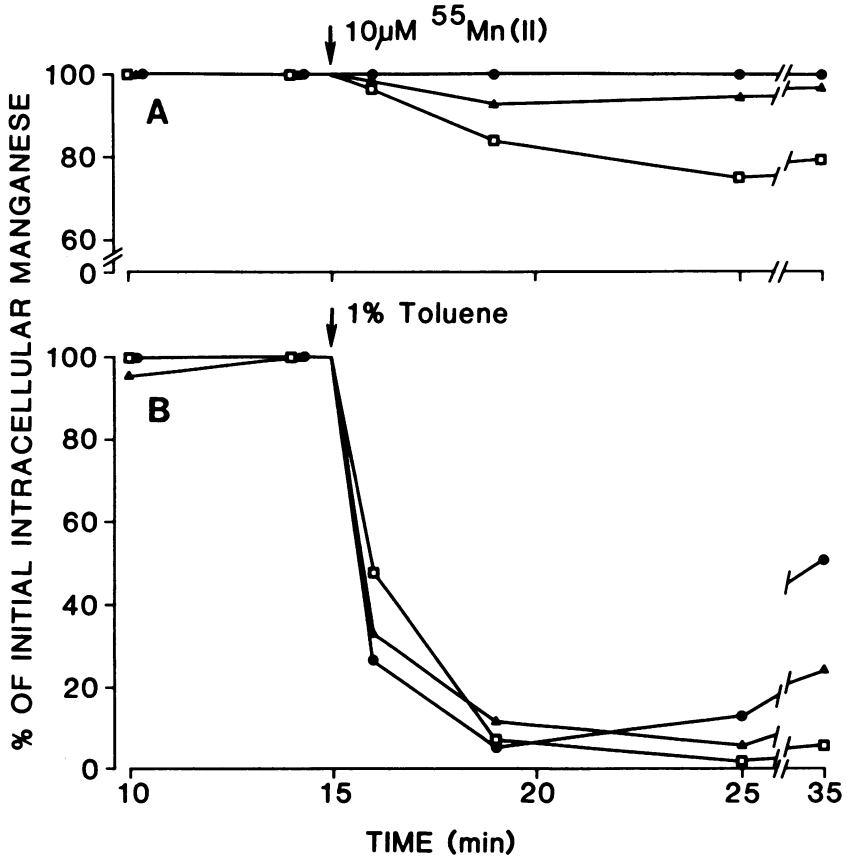

FIG. 7. The exit of $^{54}Mn(II)$ from cells with different total manganese contents. Cells were grown for 12 to 15 doublings in three separate APT broth cultures containing 1 (□), 6 (▲), and 56 (●) μM Mn(II), respectively. $^{54}Mn(II)$ was present in each culture at the same specific activity. Uptake assays were performed as described in the legend to Fig. 3 before and after the addition of 10 μM nonradioactive Mn(II) (A) and 1% (vol/vol) toluene (B) to the assay flasks.

Mn(II) as a protection against oxy-radical-mediated damage (2–5). However, unlike the superoxide dismutase activity found in most other microorganisms, total intracellular manganese was not greatly altered by varying the culture pO_2 or increasing intracellular O_2^- and H_2O_2 production with plumbagin (Fig. 1). In freshly broken cells manganese was in at least two forms: a large non-dialyzable fraction showing a small EPR signal at 25°C and a smaller dialyzable fraction with a large EPR signal (4, 5). It is thus possible that instead of altering the total cell manganese concentration, the organism alters the form and location of its manganese complexes in response to an oxy-radical threat. It is tempting to propose that the non-dialyzable manganese is "storage" Mn(II) associated with the polyphosphate granules and that the dialyzable manganese is metabolically more active and at least in part responsible for the darkened membrane region seen in unstained thin sections (Fig. 2A and B). Thus, the rapid decrease seen in initial Mn(II) uptake rates after only a small fraction of 32 mM manganese had accumulated in Mn(II)-starved cells may indicate repletion of and feedback from a smaller pool of metabolically active Mn(II) (Fig. 6). Only a fraction of the 32 mM intracellular manganese accumulated by *L. plantarum* is required for near-maximal growth rates and extents, further suggesting the presence of a large manganese storage component. The absence of polyphosphate granules from Mn(II)-limited *L. plantarum* cells (Fig. 2C), as well as the absence of high manganese accumulation in phosphate-limited cells (4), suggests that there may be an interesting regulatory mechanism involving intracellular phosphate and manganese levels.

The acquisition of Mn(II) by *L. plantarum* cells appears to depend directly or indirectly on the presence of a transmembrane proton gradient, as all four proton ionophores employed (2,4-dinitrophenol, TCS, CCCP, and nigericin) completely blocked binding and uptake by the cells (Table 1). Lacking a respiratory chain, *L. plantarum* presumably generates its transmembrane proton gradient via ATP hydrolysis in a proton-pumping ATPase, and thus the ability of the proton-pumping ATPase inhibitor DCCD to block the transport of Mn(II) further suggests the vital role of the transmembrane gradient. In contrast, the specific K^+ ionophore valinomycin had only a small depressive effect on Mn(II) uptake.

The absence of Mn(II) binding to the Mn(II) receptors of poisoned Mn(II)-starved cells would be expected if the exit of Mn(II) seen in Fig. 7A or in TCS-poisoned cells (data not shown) is via the Mn(II) uptake system. The "sealing" of the cells by 5% glutaraldehyde or 1% formaldehyde suggests that Mn(II) exits and enters via proteins and not via the lipid bilayer of the membrane (20, 24). The inability of trypsin or the broad specificity streptomycete protease to influence Mn(II) transport suggests deep immersion of the Mn(II) transporter in the membrane or protease hindrance by the cell wall. The initial release of most of the intracellular manganese (Fig. 7) by toluene does not indicate that all this manganese was in a soluble form. Experiments showed that when a 30 mM

solution of $MnSO_4$ in a dialysis bag was placed in water, a sharp transient acidification occurred in the bag as Mn(II) exchanged with protons. Such an acidification could be expected within the cells upon toluene damage to the membrane, releasing Mn(II) from complexes such as Mn(II)-polyphosphate. On the other hand, the ineffectiveness of 1% formaldehyde in preventing the rapid and extensive loss of manganese from toluene-treated cells (Fig. 7B) suggests toluene damages *L. plantarum*'s phospholipid bilayer, in agreement with findings in other organisms (13, 24).

The preference of the Mn(II) acquisition system of *L. plantarum* for Cd(II) was not unique (21, 27), but nonetheless somewhat surprising, as a comparison of the magnetic properties, mass, crystal and hydrated radii, electron shell configuration, and citrate complex stability constants of Mn(II) and Cd(II) shows less similarity than exists between Mn(II) and transition metal ions such as Co(II) or Fe(II). However, as hexaquo Mn(II) ions do not appear to be as available as Mn(II) complexed with or distorted by any of a number of anions, the degree of hydration and the electron cloud shape of the Mn(II) recognized by the cell Mn(II) uptake system may be very different from those of free Mn(II).

The results in Table 2 indicate that for optimal Mn(II) uptake, any one of a number of anions should be present. Since Mn(II) was added to these assays at 0.1 μM and the cells were grown in 17 mM citrate and phosphate before the assay, even the washed cells in citrate–phosphate-free buffer would still be exposed to more than stoichiometric amounts of these anions, and hence their requirement is apparently not for a symport process. The marked improvement in Mn(II) acquisition afforded at pH 5.5 by the presence of citrate and related natural tricarboxylic acids is interesting, as this pH is typical of the fermenting plant material from which *L. plantarum* is commonly isolated. The inability of the related tri-, di-, and monocarboxylic acids to facilitate Mn(II) acquisition at pH 5.5 suggests that there is specificity to the role played by citrate and related compounds and that they serve a functional role for *L. plantarum* in vivo.

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